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Bactericidal Mode of Action of Plantaricin C

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Plantaricin C is a bacteriocin produced by *Lactobacillus plantarum* LL441 that kills sensitive cells by acting on the cytoplasmic membrane. In contrast to its lack of impact on immune cells, plantaricin C dissipates the proton motive force and inhibits amino acid transport in sensitive cells. In proteoliposomes, plantaricin C dissipates the transmembrane electrical potential, and in liposomes, it elicits efflux of entrapped carboxy-fluorescein. It is concluded that plantaricin C is a pore-forming bacteriocin that functions in a voltage-independent manner and does not require a specific protein receptor in the target membrane.

Bacteriocins produced by lactic acid bacteria have attracted much attention in the last few years because of their potential use as fermented food preservatives. This perspective is fueled by the commercial use of the lantibiotic nisin, which is a metabolic product of some *Lactococcus lactis* strains (2). It is assumed that since lactic acid bacteria occur naturally in many, if not all, food fermentations, their bacteriocins will be easily accepted as additives by health authorities and, more importantly, by consumers. However, it is probable that the use of pure bacteriocins as food preservatives will be preceded by the inclusion of bacteriocin producers in starters since this might be considered less artificial.

In southern Europe many local, naturally ripened cheeses are made by traditional methods, in many cases without the addition of starters. These cheeses constitute a presumptively rich source of bacteriocinogenic lactic acid bacteria, which, once isolated, could be included in tailored starters, both as promoters of the transformation of the raw materials and as preservatives of their sanitary quality. With this in mind, we started to screen the microbiota present in the artisan cheeses made in the vicinity of Oviedo, Spain. A strain of *Lactobacillus plantarum* that produces a 3.5-kDa bacteriocin, designated plantaricin C, was isolated. Plantaricin C has several interesting features, such as a fairly wide spectrum of action against most of the gram-positive bacteria that are usually associated with cheese spoilage, resistance to environmental conditions, such as freezing, boiling, and pH changes, and a high specific activity (8).

Plantaricin C is bactericidal for sensitive cultures and, in some cases, induces lysis of cells. Lysis is accompanied by the immediate cessation of macromolecule synthesis and the release of uridine previously accumulated in the cytoplasm of susceptible cells (8). In this paper we present evidence that the primary target of plantaricin C is the plasma membrane, in which plantaricin C forms pores that allow free interchange of solutes between the cytoplasm and the environment.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and purification of plantaricin C. *Lactobacillus plantarum* LL441 was used as the bacteriocin producer, *Lactobacillus sake* CECT 906 was used as the standard sensitive strain, and *Lactobacillus fermentum* LMG 13554 was used as a representative of the strains that lyse upon incubation with plantaricin C. Most cultures were grown in MRS broth or agar (Biokar) containing 1% (wt/vol) glucose at 37°C without aeration; the exception was *Lactobacillus sake*, which was incubated at 30°C. Plantaricin C was purified to homogeneity as described previously (8) and was stored at 4°C.

Preparation of cell suspensions. Cultures were grown aerobically to the mid-log phase (optical density at 600 nm [OD₆₀₀], 0.6), harvested by centrifugation at 4°C, washed twice, resuspended in buffer, and finally stored on ice. Unless indicated otherwise, cells were suspended in 50 mM potassium phosphate, pH 6.4. For transport and transmembrane electrical potential ($\Delta\psi$) measurements, the buffer was supplemented with 50 mM KCl and 5 mM MgSO₄. A solution with low buffering power (0.5 mM potassium phosphate [pH 6.5] containing 70 mM KCl and 1 mM MgSO₄) was used in the glycolysis assays.

Transport assays. Transport assays were performed as described previously (13). Cell suspensions were diluted in buffer to an OD₆₀₀ of 1.0 and incubated for 3 min at 30°C with glucose (final concentration, 0.5% [wt/vol]) prior to the initiation of uptake by the addition of [1-¹⁴C]2- α -aminoisobutyrate (AIB) (59 mCi/mmol; Amersham, Buckinghamshire, United Kingdom) to a final concentration of 8.5 μ M or L-[U-¹⁴C]glutamate (285 mCi/mmol) to a final concentration of 1.75 μ M. For glutamate uptake experiments, cells were preincubated with chloramphenicol (150 μ g/ml) to inhibit protein synthesis. Uptake was further analyzed by filtration on 0.45- μ m-pore-size cellulose nitrate filters (Schleicher & Schuell, Dassel, Germany) as described previously (13).

Measurement of ATP levels. Intracellular ATP levels were determined as described previously (12). To a cell suspension (OD₆₀₀, 1.0), 0.5% (wt/vol) glucose was added, and the preparation was dispensed into two tubes and equilibrated at 30°C for 3 min. Plantaricin C was added to one of the tubes, and incubation was continued. At specified times, 0.2-ml portions of the suspensions were removed and filtered through 0.45- μ m-pore-size cellulose nitrate filters, and the ATP contents of each filtrate and filter were determined. Cells were extracted by placing the filters into vials containing 0.3 ml of ice-cold 14% perchloric acid and 9 mM Na-EDTA. After 40 min, the suspension was neutralized with 0.1 ml of 1 M KOH–1 M KHCO₃, centrifuged to eliminate cellular debris, and stored at –80°C. Samples were diluted 40-fold in assay buffer (40 mM Tris-H₂SO₄, 2 mM Na-EDTA; pH 7.75), and 0.1-ml portions of the resulting solutions were mixed with luciferin-luciferase reagent (12). Measurements were determined with an Aminco Chem-Glow photometer. The assays were calibrated by using ATP solutions of known concentrations.

Measurement of the $\Delta\psi$. The $\Delta\psi$ (inside negative) in intact cells was determined from the distribution of the lipophilic cation tetraphenylphosphonium (TPP⁺) by using a TPP⁺-selective electrode (19) or by quenching the potential-sensitive fluorescent probe 3',3'-dipropylthiadicarbocyanine. Fluorescence values were determined with a Perkin-Elmer model LS 50 spectrofluorimeter at 30°C with continuous stirring. The excitation and emission wavelengths were set at 622 and 670 nm, respectively. Each cell

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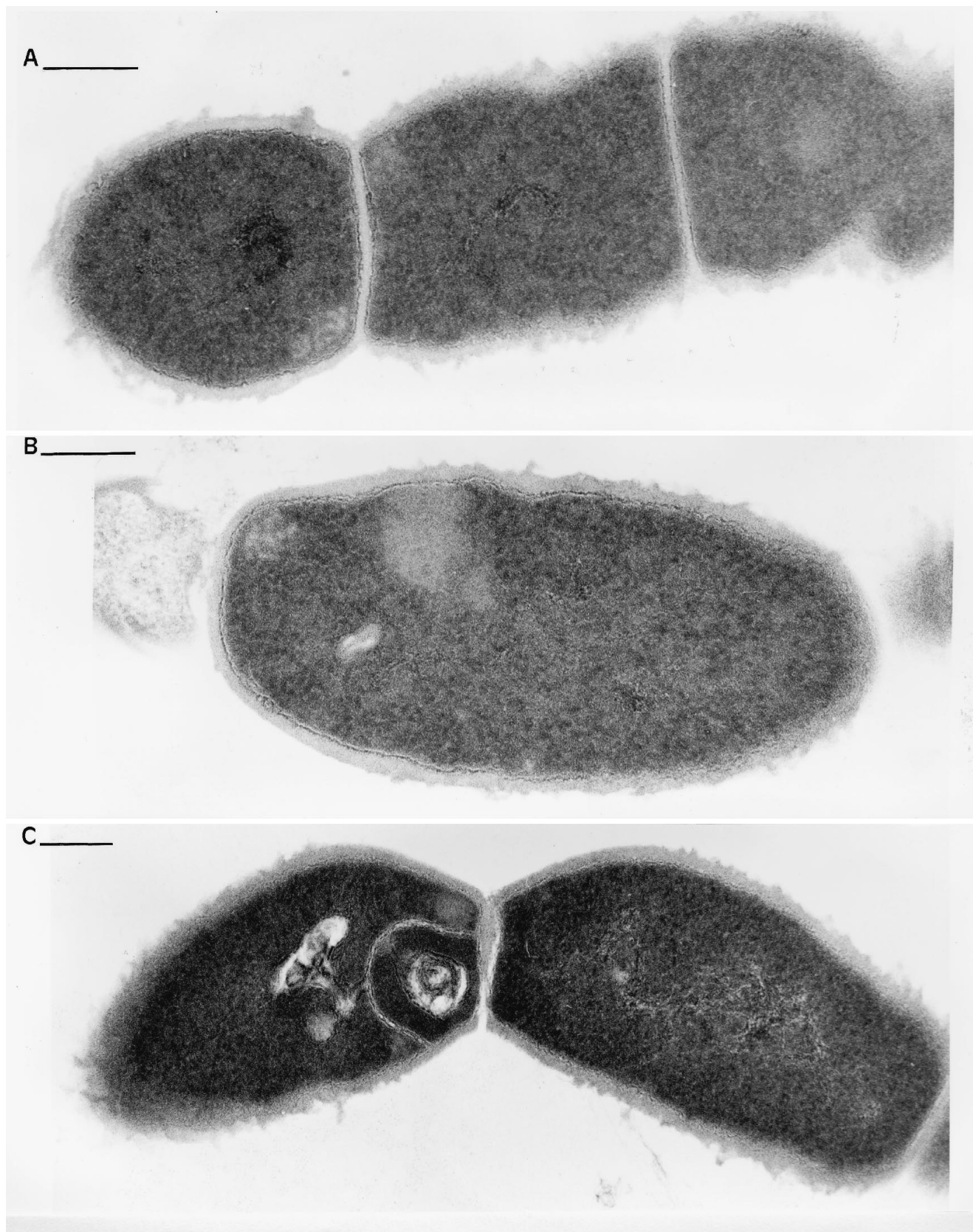


FIG. 1. Morphology of exponentially growing cells of *Lactobacillus sake* CECT 906 (A through C) and *Lactobacillus fermentum* LMG 13554 (D through F), in the presence (B, C, E, and F) and absence (A and D) of plantaricin C (1,000 AU/ml). Bars = 200 nm.



FIG. 1—Continued.

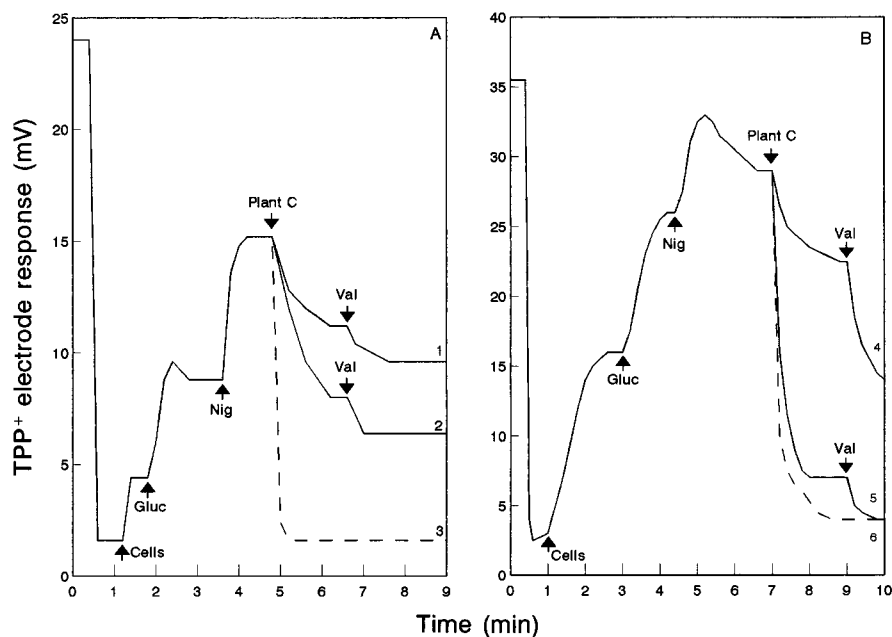


FIG. 2. Effect of plantaricin C on the $\Delta\psi$ of glucose-energized cells of *Lactobacillus sake* CECT 906 (A) and *Lactobacillus fermentum* LMG 13554 (B). At the times indicated by the arrows, cells, 0.5% (wt/vol) glucose (Gluc), 1 μ M nigericin (Nig), plantaricin C (Plant C), and 1 μ M valinomycin (Val) were added. Plantaricin C was added at concentrations of 200 AU/ml (trace 1), 400 AU/ml (traces 2 and 4), and 1,600 AU/ml (trace 5). For traces 3 and 6, valinomycin was added as a control.

suspension was diluted to an OD₆₆₀ of 1.0, and TPP⁺ was added to a final concentration of 4 μ M or 3',3'-dipropylthiadicarbocyanine was added to a final concentration of 3 μ M. Experiments were performed in the presence of nigericin (0.25 μ M) to prevent the generation of a transmembrane pH gradient (Δ pH) (3).

Measurement of the Δ pH. The Δ pH (inside alkaline) was calculated from the transmembrane distribution of benzoic acid (13, 15). Cell suspensions (2 mg of protein per ml) were supplemented with 0.5% (wt/vol) glucose and 16 μ M [¹⁴C]benzoic acid (10.9 mCi/mmol; Amersham) and incubated at 30°C for 15 min. Each cell suspension was divided into two equal fractions, to which either plantaricin C or an equal volume of solvent was added. At different times, 600- μ l samples were removed and centrifuged through a layer of silicone oil into a 10% solution of perchloric acid. Pellet and supernatant fractions were neutralized (15), and counts were determined by liquid scintillation spectrometry.

Measurement of glycolytic activity. Glycolytic activity was determined by monitoring the changes in the pH values of cell suspensions in the presence or absence of plantaricin C. Cells (OD₆₆₀, 1.0) were suspended in a solution with low buffering power (see above) containing 0.5% (wt/vol) glucose or 0.5% (wt/vol) maltose, and the change in pH was monitored continuously with a pH electrode.

Preparation of proteoliposomes and generation of a proton motive force (Δ p). Cytochrome *c* oxidase proteoliposomes composed of a mixture of *Escherichia coli* phospholipids and phosphatidylcholine (3:1) were prepared as described previously (4). The proteoliposomes were energized by adding 10 mM ascorbate (pH 6.0), 200 μ M *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, and 10 μ M cytochrome *c* (4).

Release of CF from liposomes. Liposomes loaded with 50 mM 5(6)-carboxyfluorescein (CF) were prepared from *E. coli* lipids as described previously (7). At this concentration the fluorescence of CF is almost completely self-quenched. Liposomes (70 μ g of lipid per ml) were suspended in 50 mM potassium phosphate (pH 6.0) at 25°C. After plantaricin C was added, the release of liposome-encapsulated CF (which resulted in the reversal of fluorescence self-quenching) was measured with a Perkin-Elmer model LS50 spectrofluorimeter by using excitation and emission wavelengths of 430 and 520 nm, respectively. The maximal fluorescence was determined after 0.2% (vol/vol) Triton X-100 was added to release all of the CF.

Protein determination. Protein concentrations were determined by the method of Lowry et al. (11), using bovine serum albumin as the standard.

Electron microscopy. Samples were taken from exponentially growing sensitive cultures before plantaricin C was added and 10 min after plantaricin C (200 activity units [AU]/ml) was added. Cells were harvested by centrifugation, washed, and fixed in sodium cacodylate buffer containing 3% (vol/vol) glutaraldehyde for 1 h at 4°C. The preparations were postfixed in 1% (wt/vol) OsO₄-0.5% (wt/vol) K₂Cr₂O₇ in sodium diethylbarbiturate-acetate buffer (16) for 2 h at

room temperature, and this was followed by overnight incubation in 1% (wt/vol) uranyl acetate. Samples were dehydrated with ethanol and embedded in Epon 812. Thin sections were stained with lead citrate (14) and observed with a Philips model CM10 electron microscope at 80 kV.

RESULTS

Ultrastructural changes induced in sensitive cultures by plantaricin C.

To study the mode of action of the bacteriocin

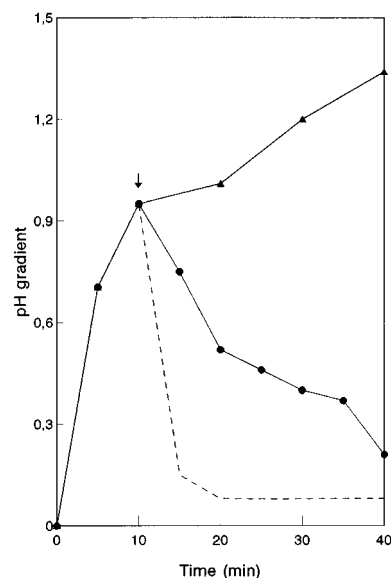


FIG. 3. Effect of plantaricin C on the generation of a Δ pH in *Lactobacillus fermentum* LMG 13554. The generation of a Δ pH upon the addition of glucose was monitored in the absence (▲) and in the presence (●) of plantaricin C (800 AU/ml), which was added at the time indicated by the arrow. The dashed line indicates the results obtained when nigericin (1 μ M) was added as a control.

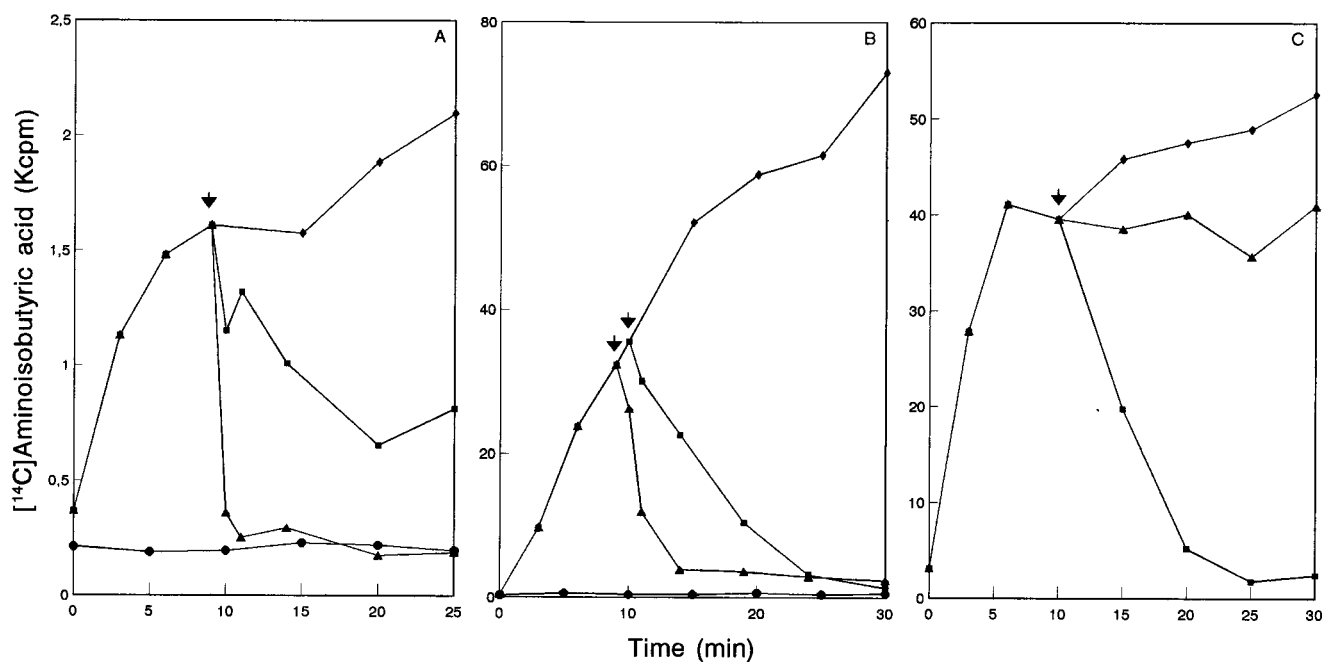


FIG. 4. Effect of plantaricin C on the uptake of AIB by glucose-energized cells of *Lactobacillus sake* CECT 906 (A), *Lactobacillus fermentum* LMG 13554 (B), and *Lactobacillus plantarum* LL441 (C). Symbols: ◆, no addition (control); ▲, plantaricin C (1,000 AU/ml) added at the time indicated by the arrow; ■, valinomycin (1 μ M) and nigericin (1 μ M) added at the time indicated by the arrow; ●, plantaricin C (1,000 AU/ml) added 2 min prior to the initiation of uptake by the addition of AIB.

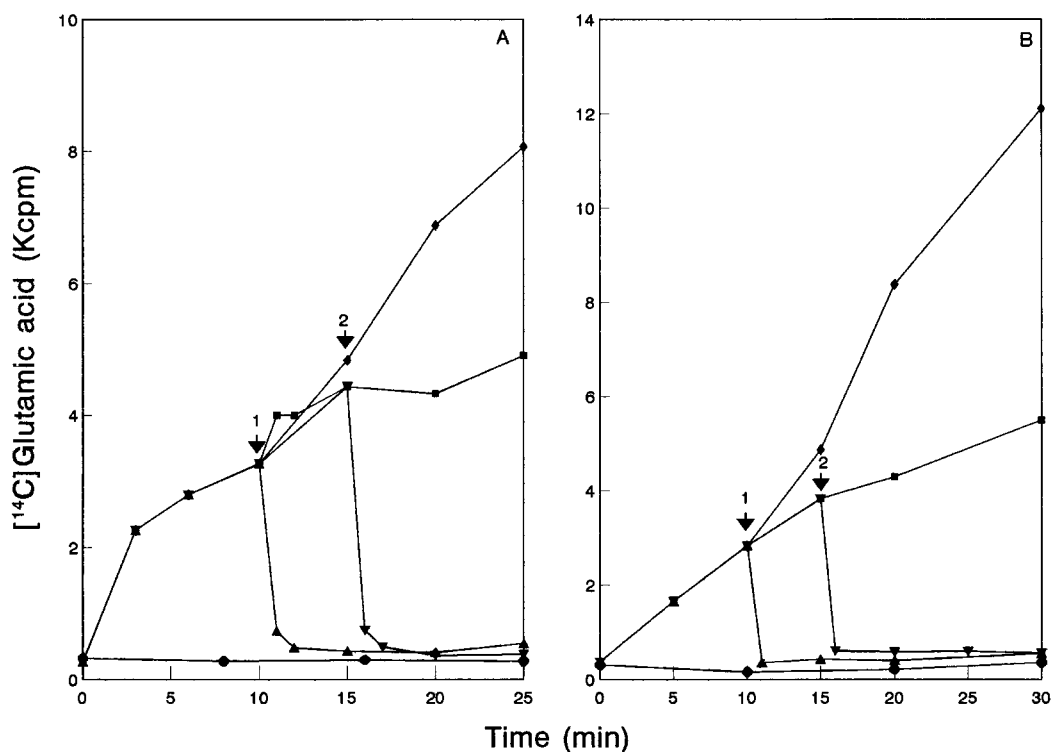


FIG. 5. Effect of plantaricin C on the uptake of glutamate by glucose-energized cells of *Lactobacillus sake* CECT 906 (A) and *Lactobacillus fermentum* LMG 13554 (B). Symbols: ◆, no addition (control); ▲, plantaricin C added at the time indicated by arrow 1; ■, valinomycin and nigericin added at the time indicated by arrow 1; ▼, valinomycin and nigericin added at the time indicated by arrow 1, followed by the addition of plantaricin at the time indicated by arrow 2; ●, plantaricin C added 2 min prior to the initiation of uptake by the addition of glutamate. Plantaricin C was used at concentrations of 1,000 AU/ml (A) and 2,000 AU/ml (B). Valinomycin and nigericin were each used at a concentration of 1 μ M. Cells were treated with chloramphenicol (150 μ g/ml) to inhibit metabolism of glutamate.

plantaricin C, two types of sensitive cells, *Lactobacillus fermentum* LMG 13554 cells (bacteriolytic effect) and *Lactobacillus sake* CECT 906 cells (bactericidal effect only) (8), and immune *Lactobacillus plantarum* LL441 cells were compared. Exponentially growing cells had the typical structure of gram-positive bacteria (Fig. 1A and D), with a thick, uniform wall to which the cytoplasmic membrane tightly adhered and a granulated cytoplasm. Mesosomes or other intracytoplasmic membrane formations were not observed. Plantaricin C induced a dramatic change in these cells. As early as 10 min after plantaricin C was added, huge mesosome-like membranous formations were observed protruding into the cytoplasm, indicating that the structure of the cytoplasmic membrane was severely affected by the bacteriocin (Fig. 1B, C, E, and F). In *Lactobacillus fermentum* cells changes were also observed at the level of the cell wall, as shown by the formation of an electron-transparent layer between the plasma membrane and the outer wall layer. Since such changes were not observed in the cell wall of *Lactobacillus sake*, which does not lyse when plantaricin C is added (8), it seems likely that the destruction of the peptidoglycan layer in *Lactobacillus fermentum* is a secondary effect of the bacteriocin.

Plantaricin C dissipates the $\Delta\psi$ of sensitive cells. The ultrastructural changes induced by plantaricin C in sensitive cells suggested that the target of plantaricin C is the plasma membrane. Since the membrane plays an essential role in energy transduction, the effects of plantaricin C on the components of $\Delta\psi$, $\Delta\psi$, and ΔpH were measured in sensitive and immune cells before and after treatment with the bacteriocin. The $\Delta\psi$ was determined quantitatively from the transmembrane distribution of the lipophilic cation TPP⁺. Upon addition of plantaricin C, the $\Delta\psi$ of energized *Lactobacillus sake* (Fig. 2A) or *Lactobacillus fermentum* (Fig. 2B) cells was partially dissipated, as shown by the release of TPP⁺. The extent of dissipation increased with the amount of plantaricin C, and *Lactobacillus sake* cells appeared to be more sensitive than *Lactobacillus fermentum* cells. Plantaricin C was not as effective in dissipating the $\Delta\psi$ as valinomycin, a K⁺ ionophore. On the other hand, addition of valinomycin to cells which were treated with plantaricin C did not lead to a complete collapse of the $\Delta\psi$, indicating that the bacteriocin might interfere with the insertion of the ionophore into the lipid bilayer and/or with the efflux of K⁺. The $\Delta\psi$ of the immune *Lactobacillus plantarum* cells was not affected by plantaricin C, while valinomycin completely abolished the $\Delta\psi$ (data not shown). Similar results were obtained when the $\Delta\psi$ was monitored qualitatively with the fluorescent probe 3',3'-dipropylthiadicarbocyanine (data not shown).

The generation of the ΔpH when glucose was added was monitored by measuring the transmembrane distribution of [¹⁴C]benzoic acid. Plantaricin C (800 AU/ml) completely dissipated the ΔpH in *Lactobacillus fermentum* (Fig. 3) and *Lactobacillus sake* (data not shown).

In order to confirm that plantaricin C indeed dissipates the Δp of sensitive cells, the effect of this bacteriocin on the accumulation of the nonmetabolizable alanine analog AIB was determined. AIB is accumulated by cells in a Δp -dependent manner in symport with one proton (9). Preincubation of *Lactobacillus sake* or *Lactobacillus fermentum* cultures with plantaricin C for 2 min completely blocked the uptake of AIB, while plantaricin C induced the immediate release of AIB that had previously been accumulated (Fig. 4A and B). Similar to its effect on the Δp , plantaricin C more effectively released AIB in *Lactobacillus sake* cells than in *Lactobacillus fermentum* cells. Moreover, plantaricin C was more effective than the ionophores valinomycin (1 μM) and nigericin (1 μM). *Lacto-*

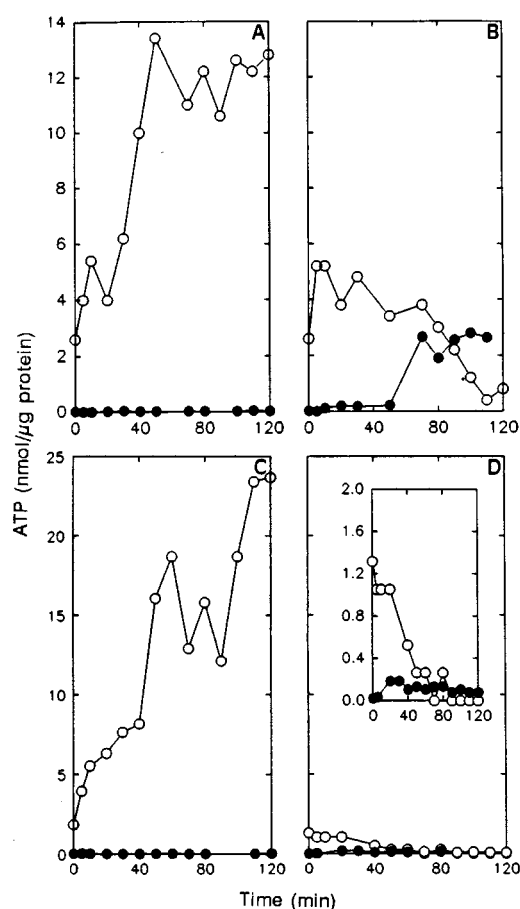


FIG. 6. Effect of plantaricin C on the intracellular (○) and extracellular (●) ATP levels in cells of *Lactobacillus sake* CECT 906 (A and B) and *Lactobacillus fermentum* LMG 13554 (C and D). (A and C) Controls without any addition. (B and D) Cells treated with 1,000 AU of plantaricin C per ml.

bacillus plantarum cells showed no efflux of AIB upon addition of plantaricin C, while an efflux was induced by valinomycin plus nigericin (Fig. 4C). These data demonstrate that plantaricin C dissipates the Δp in sensitive cells.

Plantaricin C forms pores in the cytoplasmic membrane. Plantaricin C is more effective than nigericin and valinomycin in releasing preaccumulated AIB. Therefore, efflux not only may be the result of carrier-mediated release due to the dissipation of the Δp , but also may involve non-carrier-mediated leakage of AIB through the cytoplasmic membrane. To study this possibility, the effect of plantaricin C on the transport of L-glutamate was studied. Glutamate is accumulated by cells via an ATP-driven unidirectional process which is Δp independent (13). As expected, addition of valinomycin plus nigericin to *Lactobacillus sake* and *Lactobacillus fermentum* cells did not result in an efflux of preaccumulated glutamate. However, addition of plantaricin C caused an immediate release of preaccumulated glutamate (Fig. 5A and B), indicating that plantaricin C affects the general permeability of the cytoplasmic membrane. The plantaricin C-induced release of glutamate was Δp independent, as efflux still occurred when the Δp was dissipated with valinomycin and nigericin prior to the addition of plantaricin C. *Lactobacillus fermentum* (Fig. 5B) again seemed to be less sensitive than *Lactobacillus sake* since the

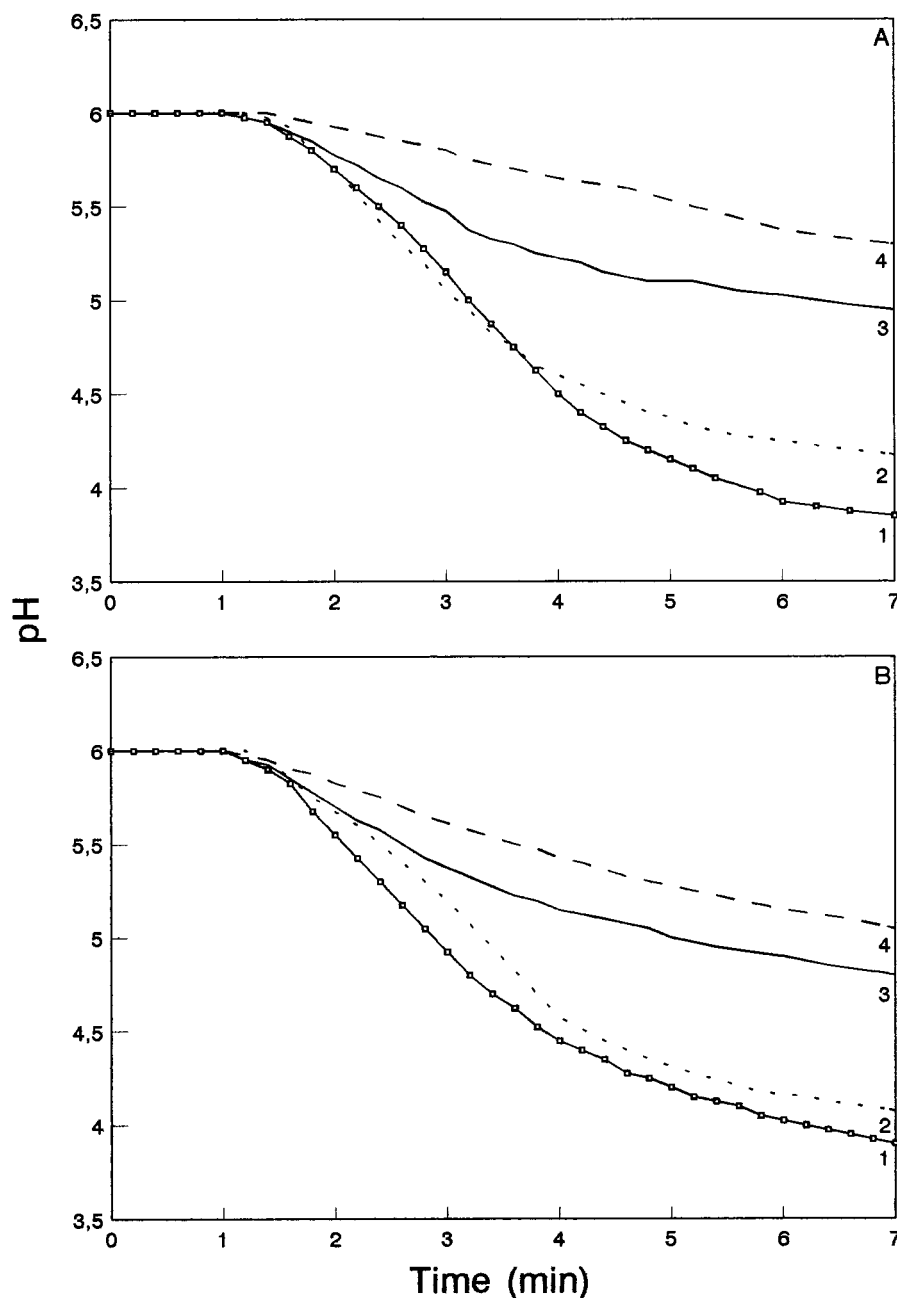


FIG. 7. Effect of plantaricin C on the glycolytic activity of cells of *Lactobacillus sake* CECT 906 (A) and *Lactobacillus fermentum* LMG 13554 (B). The following additions were made prior to the initiation of glycolysis by 0.5% (wt/vol) glucose: trace 1, no addition (control); trace 2, 1 μ M valinomycin; trace 3, 400 AU of plantaricin C per ml; trace 4, 1 μ M nigericin.

release of preaccumulated glutamate was observed only when a larger amount (2,000 AU/ml) of plantaricin C was used. In both *Lactobacillus fermentum* and *Lactobacillus sake*, the release of glutamate was immediate (Fig. 5). General release of protein was observed only at a late stage in *Lactobacillus fermentum* (data not shown), and this phenomenon correlated with the lysis of the cells. This observation suggests that plantaricin C generates pores in the cytoplasmic membrane of sensitive cells in a Δp -independent manner.

Effect of plantaricin C on the intracellular ATP levels and glycolytic activity. To determine the impact of plantaricin C

on the energetic status of the cells, the cellular ATP levels were measured. The glucose-dependent synthesis of ATP was almost immediately inhibited by the addition of plantaricin C (compare Fig. 6A and B for *Lactobacillus sake* and Fig. 6C and D for *Lactobacillus fermentum*). After longer periods of incubation, the intracellular ATP concentration dropped to very low levels, while the levels of external ATP increased. This indicates that the bacteriocin-induced ATP depletion was partially due to the release of ATP from the cells.

Since lactobacilli generate ATP by substrate level phos-

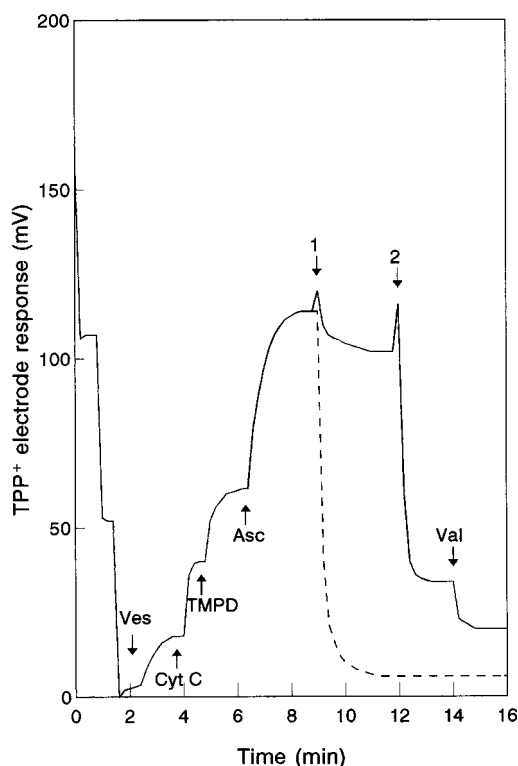


FIG. 8. Effect of plantaricin C on the $\Delta\psi$ in cytochrome *c* oxidase proteoliposomes. Proteoliposomes were energized by the sequential addition of cytochrome *c* (Cyt C), *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD), and ascorbate (Asc). At the times indicated by arrows 1 and 2, plantaricin C was added to concentrations of 200 and 1,000 AU/ml, respectively. Valinomycin (Val) was added to collapse $\Delta\psi$ completely. The dashed line shows the results of a separate experiment in which valinomycin was added. Proteoliposomes (Ves) were added at the time indicated by the arrow.

phorylation, the effect of plantaricin C on the glycolytic rate was studied by measuring the rate of acidification of the medium after the addition of a fermentable sugar (i.e., glucose) (Fig. 7). Plantaricin C caused a significant reduction in the glycolytic rate (about 50%) of glucose-energized cells of *Lactobacillus sake* (Fig. 7A) and had a smaller effect on *Lactobacillus fermentum* cells (Fig. 7B). Inhibition of glycolysis could be due to one or more steps of the metabolic pathway, including inhibition of the uptake of the sugar. Dissipation of the $\Delta\psi$ by valinomycin had only a minimal effect on the glycolytic activity. However, dissipation of the ΔpH by nigericin severely inhibited the rate of glycolysis. Since plantaricin C dissipates the ΔpH , the results are most easily understood in terms of inhibition of glycolysis due to a lowering of the intracellular pH.

Effect of plantaricin C on liposomes and proteoliposomes.

The results presented above indicate that plantaricin C forms pores in the cytoplasmic membranes of sensitive cells. To determine whether this bacteriocin is also active in a plain lipid bilayer, its effect on cytochrome *c* oxidase proteoliposomes that were energized by the addition of an electron donor system consisting of ascorbate, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, and cytochrome *c* was analyzed. The $\Delta\psi$ was monitored by determining the distribution of TPP^+ . Plantaricin C caused a concentration-dependent dissipation of $\Delta\psi$ in these proteoliposomes (Fig. 8), suggesting that plantaricin C is also able to form pores in liposomal membranes. This suggestion is

supported by the observation that plantaricin C induced the release of CF from liposomes in a concentration-dependent manner (Fig. 9).

DISCUSSION

Plantaricin C is 3,500-Da peptide bacteriocin produced by *Lactobacillus plantarum* LL441 that is bactericidal for gram-positive bacteria and in some cases induces cell lysis (8). In the present work we demonstrated that plantaricin C primarily acts on the permeability barrier of the cytoplasmic membrane. It dissipates the Δp and interferes with Δp -requiring processes. Plantaricin C also induced the release of ATP, CF, and glutamate. We concluded that plantaricin C is a pore-forming bacteriocin.

To allow permeation of large molecules, such as ATP, it is likely that several plantaricin C molecules act together to form multi-peptide pore complexes through which hydrophilic solutes can pass. In addition, secondary release of solutes may occur when the membrane structure disintegrates. Such membrane damage is evident in electron micrographs of plantaricin C-treated cells. The activity of plantaricin C is Δp independent, since plantaricin C is able to induce the release of glutamate from cells in which the Δp has been dissipated by valinomycin and nigericin, while it causes the release of CF from liposomes in the absence of a Δp . In this respect, plantaricin C seems to differ from colicins and some lantibiotics (5, 10, 17, 18). These peptides require a threshold Δp of correct polarity (negative and alkaline inside) in order to form a nonselective pore. This requirement has been explained on the basis of the cationic nature of the peptides, their interaction with acidic phospholipids, and the energy that is required to drive membrane insertion. Plantaricin C is also cationic, but the positive charges are concentrated in the amino terminus (lysine residues at positions 1, 2, 4, and 5), while the net charge of the remainder of the molecule is 0 or 1. The positively charged amino terminus may be needed for an electrostatic interaction with acidic phospholipids, as suggested for nisin (5), which would then be followed by membrane insertion of the uncharged portion of the bacteriocin. To understand the relatively wide spectrum of action (8), it will be important to analyze the lipid requirement for plantaricin C action in liposomes.

Plantaricin C-induced lysis seems to be a secondary effect of the bacteriocin, since it is observed only with some sensitive strains. Lysis appears to be preceded by depolymerization of the peptidoglycan layer (which results in a lower electron density in electron micrographs). This may be indirectly due to activation of autolysins (1, 6) and may explain why the overall effect of plantaricin C is more severe with *Lactobacillus fermentum* than with *Lactobacillus sake*, even though *Lactobacillus sake* is more sensitive than *Lactobacillus fermentum*. The lytic effect could potentially be used to accelerate cheese ripening by facilitating the release of cytosolic enzymes.

On the basis of the data presented here, we propose that plantaricin C is a pore-forming bacteriocin that causes the dissipation of the Δp and the release of intracellular molecules. Consequently, many vital Δp -dependent processes, such as the accumulation of ions and metabolites, are blocked, while the lowering of the intracellular pH reduces the glycolytic rate and thereby lowers the production of ATP. Moreover, the release of ATP and unabated hydrolysis of ATP by the proton-pumping ATPase result in a rapid depletion of the intracellular ATP pool. These effects cause growth inhibition and result in the ultimate death of plantaricin C-sensitive cells.

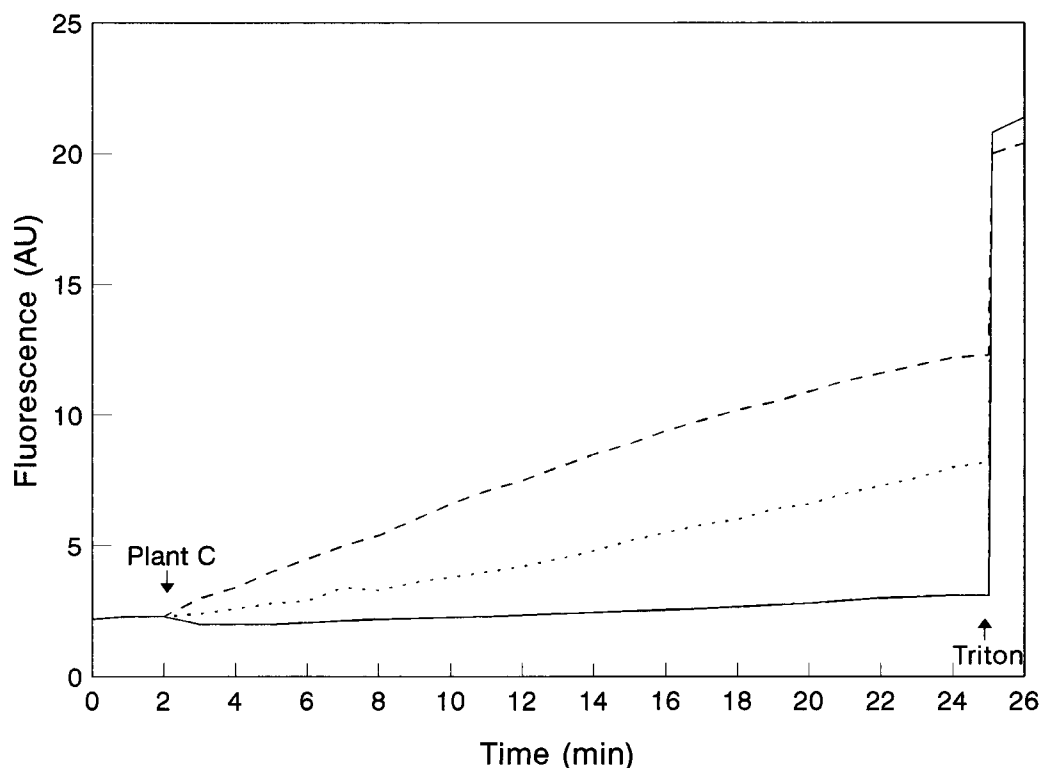


FIG. 9. Plantaricin C-induced release of CF from liposomes. At the time indicated by the arrow, plantaricin C (Plant C) was added at concentrations of 0 AU/ml (—), 400 AU/ml (· · ·), and 800 AU/ml (---). Finally, 0.1% (vol/vol) Triton X-100 was added to release the CF.

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